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Cellular Model Based on Laser Microsurgery of Cell Spheroids to Study the Repair Process

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Abstract—In this study, modern techniques of laser microsurgery of cell spheroids have been used to develop a new simple, reproducible model for studying the mechanisms of repair and regeneration in vitro. Nanosecond laser pulses were applied to perform a microdissection of the outer and the inner zones of the spheroids from dermal fibroblasts. To achieve effective dissection and preservation of spheroid viability, the optimal parameters were chosen: 355 nm wavelength, 100 Hz frequency, 2 ns pulse duration, laser pulses in the range of 7–9 μJ. After microdissection, we observed injury of the spheroids : the edges of the wound surface opened and the angular opening reached a value of more than 180°. As early as during the first hour after spheroid microdissection with laser radiation, the surviving cells changed their shape: cells on the spheroid surface and directly in the damaged area became rounded. One day after microdissection, the structure of the spheroids began to partially recover, the cells in the surface layers began to take the original flattened shape; debris of dead damaged cells and their fragments was gradually cleared from the spheroid composition. In the proposed model, the first data on stimulation of structure recovery of injured spheroids from dermal fibroblasts with a P199 synthetic polypeptide, which is used in cosmetology for the initiation of antiaging and regenerative effects in the skin, were received. After microdissection, recovery of the spheroids structure with a few surface layers of flattened imbricated arranged cells and polygonal cells of the inner zone in the presence of P199 peptide was faster than in the control group, and was completed within 7 days, presumably due to the remodeling of the survived cells.

Keywords: laser microsurgery, fibroblast spheroids, nanosecond laser scalpel, repair of cell spheroids

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INTRODUCTION

Regeneration is the restoration of the lost parts of the body that occurs after injury at different levels of the organization: cellular, tissue, organ, and whole-body. Despite the conservatism of general mechanism of regeneration in evolution, mammals lost the ability to fully restore vast areas of the body after injury (Bely, 2010). In recent years, cells grown in vitro have been increasingly used as a model not only for research of cytotoxicity or the effectiveness of immunobiological products, drugs, or cosmetics (Kolokol'tsova et al., 2014) but also for the study of regeneration and reparation processes. Since proliferative activity and physiological properties of the cells in 3D systems are similar to in vivo conditions, researchers are actively starting to use 3D cultivation instead of 2D (Haycock, 2011). In 3D cultures, cells grow interacting with the environment (other cells, extracellular matrix and the external environment) in all three dimensions (Pampaloni et al., 2013; Antoni et al., 2015; Kubatiev et al., 2015). Cellular spheroids are one of the most common options for 3D simulation ex vivo. They represent 3D self-organizing spherical clusters of cells. The organization of spheroids is similar to the organization of tissues in vivo with a complex of intercellular contacts and the contacts with the newly synthesized extracellular matrix (Lin and Chang, 2008). Many cell types have a natural tendency to spontaneously aggregate. General patterns of the formation of spheroids from cells of epithelial and mesenchymal phenotypes have already been described (Repin et al., 2014).

Studying the mechanisms of wound healing is one of the challenging issues nowadays, where spheroids can be applied as an in vitro model system. Using in vitro monolayer cultures as a model system allows for study of only specific parameters of cell behavior,

such as the rate of migration and proliferation, but it does not allow for evaluating 3D intercellular interactions or for interactions of cells with the extracellular matrix. Therefore, wound-healing simulation is now performed mainly using animals in vivo or using organotypic explant cultures ex vivo (Gottrup et al., 2000; Antoni et al., 2015). The search for simple, reproducible model systems for studying mechanisms of regeneration and repair continues. Repair of cellular spheroids after damaging effects could be one of such models. In this paper, we propose to use the technique of laser microsurgery to model spheroid damage.

Laser microdissectors based on pulsed lasers have been widely used in modern biology and medicine. They enable microsurgery both at the tissue and cellular and even at the subcellular levels (Magidson et al., 2007). The most common femtosecond laser sources provide high spatial and temporal resolution and have high penetration depth. Femtosecond laser systems have been demonstrated to be successful for dissection of the nuclei and the inactivation of cell organelles (Heistercamp et al., 2005; Shen et al., 2005) and selective delivery of extracellular substances into the cells—optoinjection and transfection (Stevenson et al., 2007; Uchugonova et al., 2008; Il'ina et al., 2013, 2014). In recent years, the possibility of successful microsurgery of intracellular structures has been demonstrated using nanosecond (Khodjakov et al., 2004; Magidson et al., 2007) and picosecond laser systems (Colombelli et al., 2005) with accuracy comparable to that of femtosecond systems (Sacconi et al., 2005).

In addition to 2D structures (cell monolayers), laser microsurgery techniques have also been tested on 3D objects—embryos of different organisms and spheroids. In particular, laser dissectors were used for creating a hole in the oocyte zona pellucida for further in vitro artificial fertilization or polar body biopsy (Clement-Sengewald et al., 1996), for assisted hatching and dissection of trophectoderm cells during biopsy at the later stages of preimplantation development (Ilina et al., 2015, 2016). Despite the fact that embryos are 3D objects, they are convenient models for only a limited number of problems. Cellular spheroids as model objects allow us to bridge the gap between living tissue and in vitro 2D structures creating prerequisites for the development of models based on laser microsurgery of cellular spheroids. Despite this, there are currently only a few studies in which laser techniques are used for studying spheroids. These works are concerned with techniques of spheroid visualization and spheroid differentiation (Ilina et al., 2011; König et al., 2011; Bruns et al., 2012; Pampaloni et al., 2013), methods to impact and locally damage spheroids (Uchugonova et al., 2007; Kosheleva et al., 2016). At the moment, the study of characteristics of the spheroid repair process after large-scale extended injuries has not been conducted to date.

Skin is an organ with a barrier function and is frequently subjected to damaging influences (physical, mechanical, etc.) of the environment, and, therefore, has a large regenerative potential. Fibroblasts are the key cell elements that are involved in morphogenesis and dynamic remodeling of the dermis, including protein synthesis and extracellular matrix metalloproteinases (Jenkins, 2002; Makrantonaki and Zouboulis, 2007). Therefore, 3D cultures of human dermal fibroblasts have been used in model development to investigate the repair process.

Biologically active products that stimulate proliferative and synthetic activity, especially of fibroblasts, are actively used in cosmetology for repair of skin defects and in antiaging medicine. These products may be cytokines, cell extracts, or synthetic peptides. The model under development in this study can become a convenient test system for testing their biological activity. Therefore, we used a P199 synthetic peptide, which is the main component of a medical product used in cosmetology for stimulating regeneration and rejuvenation of the skin (Petrikovskii, 2012).

The aim of this study was to develop a simple reproducible model of cellular spheroids injury using laser microsurgery technique. The study was conducted on spheroids from human skin fibroblasts. A nanosecond laser dissector was used to simulate spheroid damage. This model was used in a pilot study of the influence of a P199 synthetic peptide addition on damage repair.

MATERIALS AND METHODS

Monolayer 2D Culture of Human Skin dermal Fibroblasts

Primary cultures of human fibroblasts were used in the experiment. Cells were isolated from two biopsies of human skin obtained after informed consent of the donors. All the procedures were performed in compliance with the rules of ethics, the law on health care and aseptically. Isolation of cultures was performed by mechanical disaggregation and subsequent enzymatic treatment of the biopsies with 0.25% trypsin solution. Cells were cultured on Petri dishes under standard conditions (37°C, 5% CO_2) in complete growth medium containing DMEM/F12 (1 : 1, BioloT, St. Petersburg), L-glutamine (2 mM/L, PanEco, Moscow), gentamicin (50 μg/mL, PanEco), and 10% fetal bovine serum (HyClone, United States). The complete growth medium was replaced 2–3 times a week. Cell phenotype and culture confluence were controlled under a CKX41 inverted light microscope (Olympus, Japan) with the DeltaPix Viewer digital camera for photorecording (Olympus, Japan). At 70% confluence, the cells were passaged.

For analysis of cell culture proliferation, using a Countess automatic cell counter (Invitrogen, United States), the total number of cells for 48 h of cultivation was counted in triplicate. Index of proliferation (IP)

was calculated using the formula: IP = N_{48}/N_0, where N_{48} is the number of cells after 48 h of cultivation and N_0 is that after 0 h of cultivation.

3D Cultures, Cell Spheroids

To obtain spheroids in 3D culture conditions, the suspension of fibroblasts at the fourth passage were placed in agarose plates with microwells (Microtissue, United States) at 250000 cells/mL concentration. Agarose plates were transferred to wells of 12-well plates and were cultured for 7 days under standard conditions (37°C, 5% CO_2) in complete growth medium.

In a subsequent experiment, spheroids were divided into three groups: (1) laser dissector injured spheroids, which, after the exposure, were cultured on agarose plates in the presence of the P199 peptide (15 ng/mL, PremierFarma, Moscow) for 7 days; (2) laser dissector-damaged spheroids that after the exposure were cultured on agarose plates for 7 days in complete growth medium without additives; (3) intact control spheroids, which were cultured on agarose plates in complete growth medium.

The P199 synthetic peptide was added to the growth medium in order to stimulate the repair of spheroids in the experimental group. The P199 synthetic peptide composed of 72 amino acids and has the molecular weight of 8.4 kDa was developed on the basis of similar natural proteins of the Wharton's jelly of the umbilical cord.

Nanosecond Laser Microsurgery of Cell Spheroids

Spheroids were irradiated using the Palm CombiSystem (Zeiss, Germany) nanosecond laser scalpel (355 nm wavelength, 100 Hz repetition rate, 2 ns pulse duration, maximum pulse energy of 9 μJ). Laser radiation was focused on the objects using a Zeiss Fluar micro-objective (10×, NA 0.5). PALM RoboPro software was used to control laser scalpel.

The surface and inner spheroid layers were injured with nanosecond laser pulses. The laser processing of the defined straight-line path was set manually in the software and was repeated five to eight cycles. Every subsequent cycle was characterized by the axial (along *Z* axis) laser beam focus shift to provide the dissection of the spheroid in three dimensions. A total of 25–30 spheroids were irradiated in every agarose plate, which took approximately 30 min. After irradiation, the spheroids from experimental and control groups were returned to CO_2-incubator conditions (37°C, 5% CO_2) for the further observation of the repair process.

Laser microsurgery of spheroids was performed using the equipment of the The Core Facility Center "Femtosecond Laser Center" of JIHT RAS.

Time-Lapse Microscopy

Time-lapse microscopy was performed using the PALM CombiSystem photodetection installation (Zeiss, Germany) with photorecording every 5 s for the first 5 min after irradiation. Long-term live registration was performed for 7 days after laser microsurgery under standard conditions (37°C, 5% CO_2) in a chamber of the Cell-IQ time-lapse system (CM Technologies, Finland) with photorecording every 20 min using the Cell-IQ Imagen software. The images were analyzed using the Cell-IQ Analyzer software.

Seven-day spheroids of control and experimental groups, 1 h after laser microdissection were fixed for subsequent histological analysis.

Histological Analysis

Spheroids were fixed with glutaraldehyde (1.5% solution in 0.1 M cacodylate buffer, pH 7.3, 1–2 h), then fixed with OsO_4 (1% aqueous solution, 1–2 h), dehydrated in alcohols of increasing concentration of 50°, 70°, 96° (two replacements of 5 min each), acetone (three replacements of 10 min) and embedded into the mixture of Araldite M and Araldite Hardener epoxy resins (Sigma, United States) with addition of the DMP30 catalyst (Chimmed, Moscow) and dibutyl phthalate plasticizer (Chimmed, Moscow). The samples were incubated at 60°C for 3–5 days for polymerization of the resin.

Semifine sections of 1–2 μm thickness were obtained using a Leica EM UC6 ultramicrotome (Austria), stained with methylene green for 2–3 min (Sigma, United States). Preparations were analyzed under the Olympus BX51 light microscope (Olympus, Japan) equipped with a Color View II camera. Photorecording was performed with the Cell F software.

RESULTS AND DISCUSSION

Fibroblasts isolated from human skin samples had good cultural characteristics and maintained quite high proliferative potential. Primary culture at the first passage was a heterogeneous population of cells with various shapes and sizes. The cells in the active growth phase had an average size of 180 × 12 μm as shown in the study using a microscope. After 2–3 days, the cells formed a compact uniform monolayer (Fig. 1a). During passaging, the culture became homogeneous—the cells became spindle-shaped and the sizes were 100 × 10 μm on average. The cytoplasm had typical small grains; the nuclei were oriented along the long axis of the cells containing 1–3 nucleoli. We observed characteristic elongated flattened cells arranged in parallel groups and oriented in different directions. Upon further culturing, the cells retained a high proliferation index, and the values exceeded 2.5 (table). The morphology of the obtained cultures of human dermal skin cells had a typical fibroblast

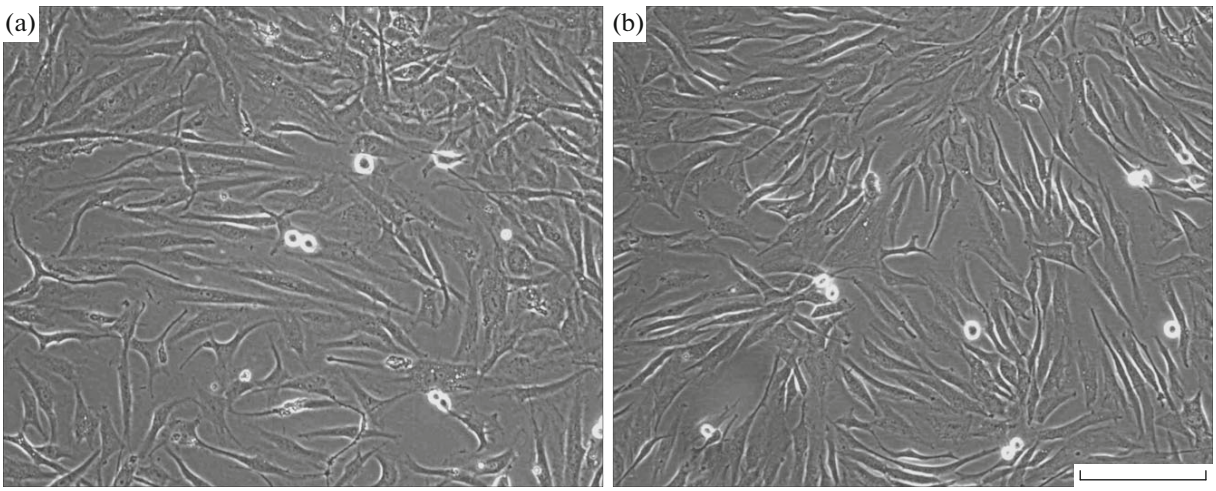

Fig. 1. Culture of human dermal fibroblasts. (a) First passage, (b) fourth passage. Light microscopy, phase contrast, the scale bar is 50 μm.

growth pattern and was represented by elongated flattened cells (Fig. 1). Fibroblasts after four passages expressed characteristic markers (cytokeratin 19, elastin, fibronectin, collagen I, III, and IV types) responsible for the main biomechanical properties of the skin, such as smoothness, toughness, and elasticity (Kozhina et al., 2015, 2016).

Under 3D conditions in agarose plates, fibroblasts formed standard viable spheroids for large-scale spheroid generation; each spheroid was formed in its own micro-well from a defined number of cells. For 7 days, dermal fibroblasts at the fourth passage successfully formed viable compact spheroids. The process of spheroid compaction of dermal fibroblasts started as early as in the first hours of 3D culturing and lasted up to 7 days. Histological analysis revealed two distinct regions in compact seven-day spheroids: 2–4 layers of elongated and flattened surface cells and an internal zone consisting of polygonal cells. Imbricated arranged cells of the surface region were in close contact with each other, whereas the inner zone was loose and polygonal cells were separated by the extracellular matrix (Fig. 3a). The average diameter of the formed spheroids was 150 μm, thus ensuring the normal diffusion of nutrients into the inner region of the spheroid.

Under 3D conditions, the cells retain the nuclear-cytoplasmic ratio and the ratio of the cytoplasm volume to the membrane area characteristic of conditions in vivo, which is required for cell functioning (Backer and Chen, 2012). This makes the cells in spheroids more stable than in regularly passaged 2D monolayer cultures (Antoni et al., 2015). When forming spheroids, the cells form intercellular contacts and create a microenvironment to maintain their native phenotype. The described structure of spheroids from human skin fibroblasts is similar to the known structure of spheroids of this type of cells (Kozhina et al., 2015) as well as mesenchymal cells of various origins (Saburina et al., 2013; Kosheleva et al., 2015).

Damage of Fibroblast Spheroids with Nanosecond Laser Scalpel

The parameters of the laser irradiation and, in particular, the energy of laser pulses were optimized to perform effective microdissection of the selected regions of the surface and inner regions of the spheroid without displacement of the object during exposure. Pulse energy was selected depending on the size of the spheroid, nonequal thickness of agarose plates, which contained spheroids and energy pulse ranged as 7–9 μJ. The process of laser microdissection and cavitation bubble formation (Fig. 2, arrow) leading to visible damage of spheroids are shown in Fig. 2. The laser exposure of spheroids lasted 5–30 s; over the next 5 min after exposure to laser pulses, we observed spontaneous opening of the wound edges and the angular opening reached a value of more than 180° (Fig. 2).

Index of proliferation of human dermal fibroblast cultures.

Biopsy of dermis	1 passage	2 passage	3 passage
1, adult donor 54 years old	2.71 ± 0.15	3.20 ± 0.25	2.67 ± 0.24
2, adult donor 52 years old	2.73 ± 0.27	2.67 ± 0.18	2.55 ± 0.25

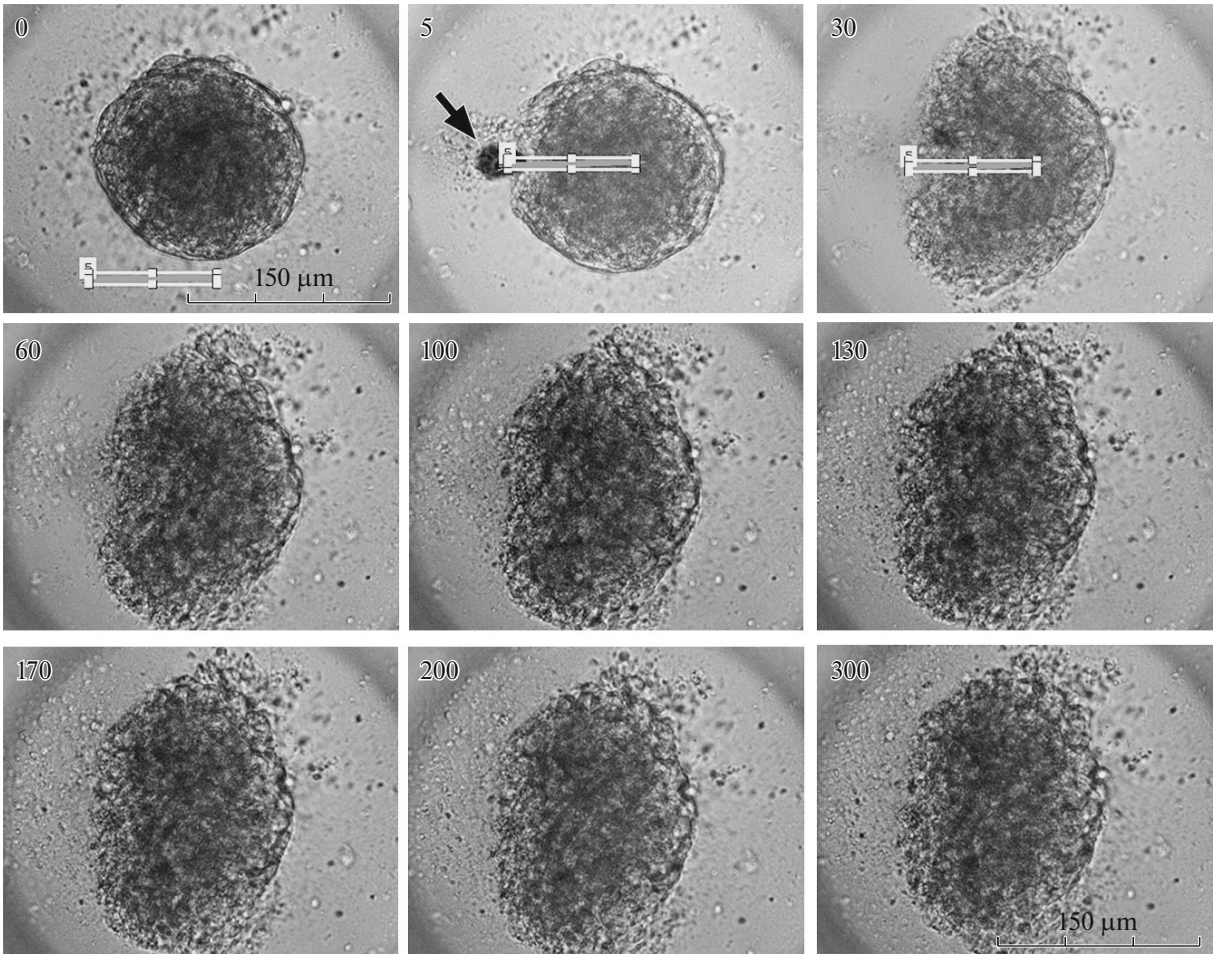

Fig. 2. Live time-lapse photorecording during the first 5 min after the exposure of seven-day human skin fibroblast spheroids to nanosecond laser scalpel impact. 0 s—general view of a spheroid before laser irradiation. The red line shows the selected trajectory of the exposure; 5–30 s—the moment of spheroid injury; 60–300 s—spontaneous disclosure of the wound edges, the increasing angular opening of the wound edges after the start of microdissection. Arrow indicates cavitation bubble. Light microscopy, phase contrast, the scale bar is 150 μm.

Fragments of dead cells appeared in the area of damage in the first minutes after exposure to laser radiation (Fig. 2). The growth of the angle of the wound edge opening was accompanied by a change in the cell morphology of the wound surface; the cells changed the shape from elongated flattened to a round one. The structure of the surface layer in the intact area remained almost unchanged and retained its integrity.

Histological analysis confirmed the described changes and showed that, within an hour after microdissection, the wound surface contained rounded cells and fragments of dead cells (Fig. 3b). In the area of damage, the original structure of spheroid was disturbed and the morphology of the surface and the inner zones became unified: the cells changed the shape to a round one. Only single cells surrounded by cellular debris retained their original morphology directly in the region of damage. Probably, processes of phagocytosis were active in the area of damage.

Plasma formation and optical-induced breakdown are the main "surgical" mechanisms that cause tissue dissection after application of nanosecond laser pulses (Rau et al., 2006; Genc et al., 2014). When the energy of pulses was slightly higher than the threshold value, optical-induced breakdown was accompanied by the formation of cavitation bubbles with the size of a few micrometers. Their emergence led to visible damage to the irradiated tissue, but it was possible to achieve localized microsurgery of the selected object due to the small size of cavitation bubbles. We thoroughly optimized the energy of nanosecond laser pulses so as to effectively perform dissection of the desired region of spheroids without compromising the integrity of spheroid in general.

Reparation of Dermal Fibroblast Spheroids after Damaging with Laser Scalpel

Long-term live observation of experimental spheroids using time-lapse microscopy for 7 days after laser

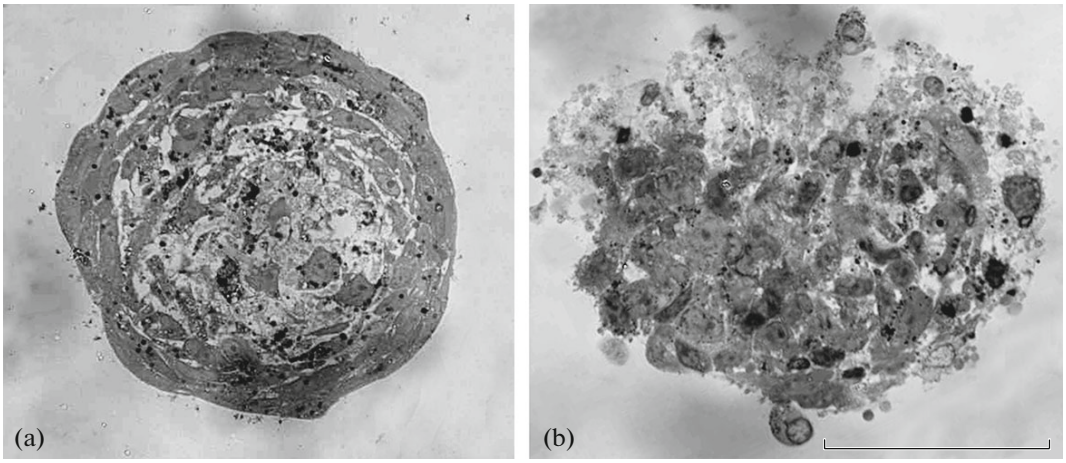

Fig. 3. Histological analysis of human skin fibroblast spheroid structure after exposure to nanosecond laser scalpel impact. (a) Intact control spheroid; (b) injured spheroid one hour after exposure to nanosecond laser scalpel. Semifine sections stained with methylene green, light microscopy, the scale bar is 50 μm.

microdissection allowed us to analyze the dynamics of the repair after exposure. Data are presented in Fig. 4. One day after the damaging exposure, the spheroid structure partially restored itself, the cells in the surface layers began to flatten; debris of dead damaged cells was gradually cleared from the spheroid.

The composition of the complete growth medium influenced the rate of repair after microdissection. Repair of spheroids was not observed for 7 days in medium without additives, debris of damaged dead cells remained, and the surface layer structure was nearly completely disrupted around the perimeter of the spheroid. The addition of the P199 synthetic peptide into the medium enhanced repair: the debris of dead and damaged cells was almost cleared as early as on the third day, the spheroid structure partially restored, and the cells in the surface zone again began to acquire a flattened shape. Full repair of the original structure of spheroids with several surface layers of flattened imbricated arranged cells and polygonal cells of the inner zone occurred by 7 days after microdissection (Figs. 4e–4h). Furthermore, it was observed that the diameter of a spheroid after repair was less than the original value.

The developed model of laser microdissection and optimally selected laser exposure conditions allowed us to damage the surface and the inner regions in a selected area of spheroid without disturbing the viability of the object in general. Under the selected regimes of laser irradiation, a lot of cellular fragments appeared in the wound area of a spheroid, which is directly related to the physical effects, while a spheroid in the uninjured area retained its initial structure. Mechanical stress relaxation plays an important role in embryogenesis, morphogenesis, and differentiation (Belousov et al., 1999; Fouchard et al., 2014). Different packing cell density of the surface and inner areas of the spheroids during microdissection led mostly to the damage of surface area cells of the experimental spheroids. Such a change could have contributed to mechanical stress relaxation and led to an increase in the angle of the wound edges opening after microdissection, which contributed to clearing of the wounds from dead cells and their fragments.

We observed recovery of the general form of spheroids in the first hours after the damage, while the internal structure of spheroids repaired gradually, and the reparative processes completed within 7 days only in the presence of the P199 synthetic peptide.

Synthetic peptides, used in cosmetology, are designed based on natural analogues and can regulate cell proliferation and migration, inflammation, angiogenesis, melanogenesis, and synthesis of extracellular matrix proteins. Furthermore, synthetic peptides are generally composed of L-amino acids, which are generally not immunogenic and are easily utilized by the body (Zhang et al., 2009). In this study, the P199 peptide was the test drug product, which is similar to proteins of the Wharton's jelly of the umbilical cord, having clinically proven effective antiaging, rejuvenating, and regenerative properties on human skin during aging and after burns (Petrikovskii, 2012). This peptide stimulates proliferation of fibroblasts and stem cells of the skin and increases the synthesis of cytokines and extracellular matrix components by human skin dermal cells (Shekhvatova et al., 2013; Yutskovskaya and Danilova, 2014). Balance of well proliferating stem cells and niche-forming components of a population is important for colony formation in fibroblast cell culture (Yegorov et al., 2007). The capacity of cells to form spheroids is a unique simple test system for the determination of cell maturity and evaluation of drug influence. Formation of spheroids requires a certain level of expression of fibronectin and integrin $\alpha 5$ and $\beta 1$ by fibroblasts (Salmenperä et al., 2008). The P199 peptide restored the ability to form spheroids in the

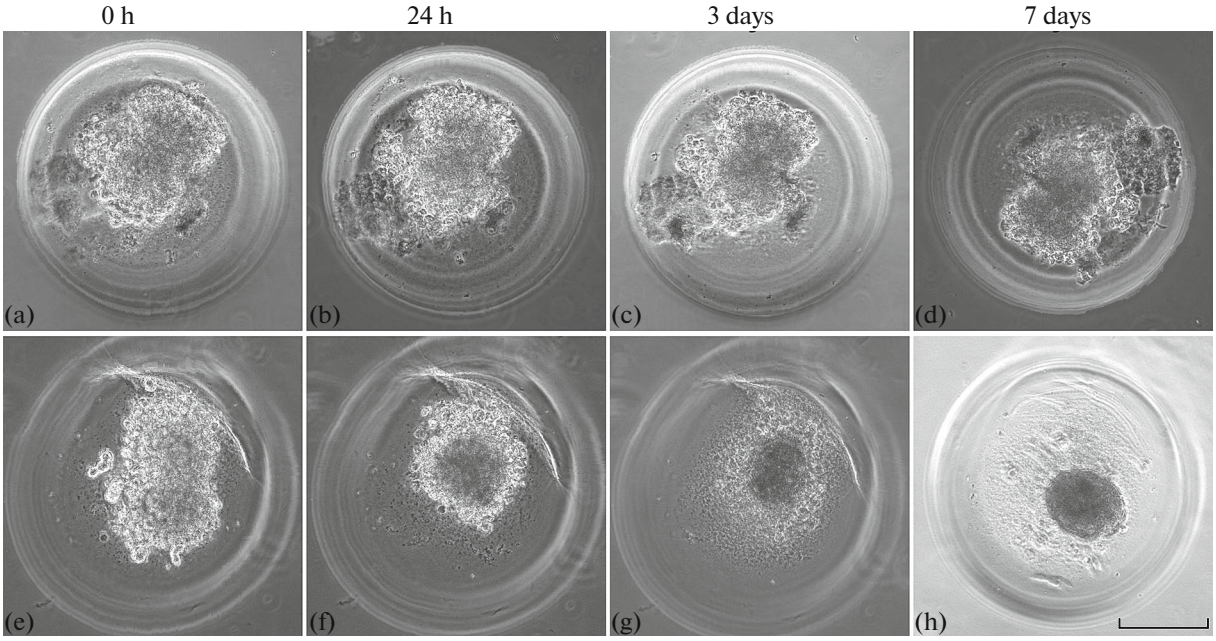

Fig. 4. Long life time-lapse recording during seven days after four-passage fibroblast spheroid microdissection with nanosecond laser scalpel. 0, 24 h, 3 and 7 days after exposure. (a–d) Spheroids after damage were cultured in medium alone, (e–h) after damage spheroids were cultured in medium supplemented with the P199 oligopeptide. Light microscopy, phase contrast, the scale bar is 100 μm.

culture of senescent dermal fibroblasts at 18 passages by stimulating the synthesis of sufficient amounts of extracellular matrix components (elastin, collagen IV type, fibronectin) for establishing intercellular contacts and restoration of mesenchymal-epithelial plasticity of cells (Kozhina et al., 2016). Probably, in our pilot study of fibroblast spheroid repair after microdissection, addition of P199 peptide stimulated the restoration of the structure mainly due to the regulation of the synthesis of extracellular matrix components.

Importantly, after microdissection, the diameter of repaired spheroids decreased versus the original diameter, the wound edges did not close, and the defect was apparently filled with intact cells of the inner zone. Therefore, it can be assumed that the spheroid repair of dermal fibroblasts after laser microdissection occurred due to remodeling of the existing viable cells rather than through proliferation.

CONCLUSIONS

Repair and restoration of tissues play an important role in the normal functioning of the body. Studying of repair in vitro using monolayer cell cultures allows us to evaluate only proliferation and migration of cells and only in one plane. Models that take into account 3D complex interactions of cells with each other and with the extracellular matrix are required for a detailed understanding of regeneration processes. The cellular spheroids can be considered analogous to avascular tissues; they are an adequate in vitro model for studying wound healing. We have proposed and developed a new simple reproducible model for studying the mechanisms of wound healing in 3D in vitro cell spheroids using laser microdissection. The developed model of laser microdissection and optimally selected laser exposure conditions allowed us to damage the surface and the inner area in the desired region of the spheroid without compromising its viability in general. In the proposed model, we have received the first data on stimulation of fibroblast spheroid structure repair with the P199 synthetic peptide, which initiates anti-aging and regenerative effects in the skin.

Model of spheroids damage based on laser microsurgery opens up new possibilities for studying regeneration mechanisms and the role of spheroid-forming cells in repair.

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